Exosome-Based Transfection

Honors Research Thesis

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Abstract

Exosomes are mammalian extracellular vesicles that are involved in intracellular communication. The discovery of RNA in exosomes established their capacity to function as RNA delivery vehicles in gene therapy applications. The main obstacle to the gene therapy approach is finding suitable vehicles to deliver therapeutic genetic molecules. Conventional delivery methods employ viruses or liposomes, however, the clinical applications of these vehicles are limited by shortcomings such as cytotoxicity and immunogenicity. On the other hand, exosomes could represent a more effective delivery vehicle because they are natural transporters of RNA. The purpose of the research presented in this thesis was to investigate the transfection ability and cytotoxicity of exosomes. Exosomes were used to transfect mouse embryonic fibroblasts (MEFs) *in vitro* with mRNA for *Ascl1*, *Brn2* and *Myt1l* (ABM), a combination of transcription factors that reprograms fibroblasts into neurons. The exosomes were prepared by isolating them from the supernatant of MEFs transfected with ABM plasmids using bulk electroporation (BEP). Exosome cytotoxicity was compared to that of a commercial liposome formulation by incubating them with MEFs and determining cell viability. The results indicate that exosomes successfully transfected MEFs with ABM mRNA. In addition, the exosomes were less cytotoxic than the liposomes. In fact, viability of MEFs was minimally impacted by incubation with the exosomes. Further research in exosome-based transfection could one day allow researchers to harness their intrinsic properties with the purpose of delivering RNA for gene therapy.

Introduction

Extracellular vesicles have recently gained attention for their role in intercellular communication. Because they facilitate the transmission of signals between cells, extracellular vesicles have the potential to be used in the delivery of therapeutics [1]. Exosomes are nano-sized (40-100 nm) mammalian extracellular vesicles. These vesicles lack organelles but are composed of cytosol surrounded by a lipid bilayer membrane. Cells begin to form exosomes when membrane-bound proteins are endocytosed into the early endosome (EE) [2]. Then, the late endosome (LE) invaginates to form internal vesicles and multivesicular bodies (MVB) which release exosomes extracellularly upon fusion with the plasma membrane (Figure 1).

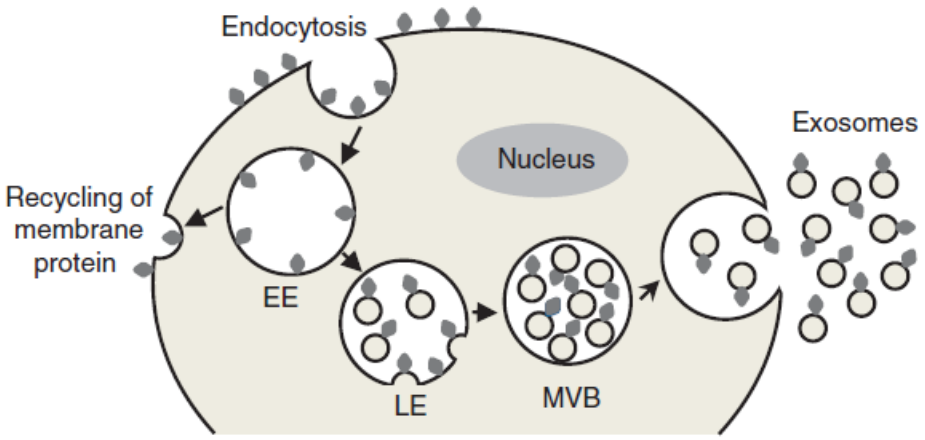

Figure 1: Illustration of exosome biogenesis and release [2].

Initially, exosomes were described as vehicles for clearing cell debris and unwanted molecules. However, in 1996 an experiment indicated that exosomes participate in antigen presentation because exosomes from B cells were shown to induce T cells [3]. In another study, exosomes derived from dendritic cells were able to trigger mice immune systems to eliminate tumors [4]. Exosomes have since been discovered to be involved in many more immunological

functions and in areas such as pregnancy, cancer and neurodegenerative diseases [5]. Besides B cells, T cells and dendritic cells, exosomes have been shown to be secreted by numerous other mammalian cell types, including tumor cells, epithelial cells and platelets [6]. Studies have also reported that exosomes are found in a range of body fluids such as amniotic fluid, blood and urine [7].

An important breakthrough in 2007 was the discovery of mRNA and microRNA in exosomes [8]. The study also revealed that the mRNA was functional because it was successfully translated into proteins *in vitro*, and demonstrated that incubation with exosomes from a murine cell line resulted in the expression of murine proteins in human cells. This breakthrough revealed that exosomes have a natural ability for the transfer of genetic material, and as such, it established their potential to function as delivery vehicles in gene therapy applications.

Gene therapy is the delivery of genetic molecules or nucleic acids for therapeutic purposes. This approach is currently the subject of substantial research on afflictions such as cancer and neurological diseases [9]. A significant obstacle to the gene therapy approach is finding an effective nucleic acid delivery vehicle to protect genetic molecules from degradation and facilitate their uptake by cells [10]. Ideally, a nucleic acid delivery vehicle should be capable of targeting the specific cell type of interest and delivering the cargo intracellularly without inducing an immune response or producing harmful side effects [11]. The most prevalent delivery methods in gene therapy are viruses and liposomes (Figure 2).

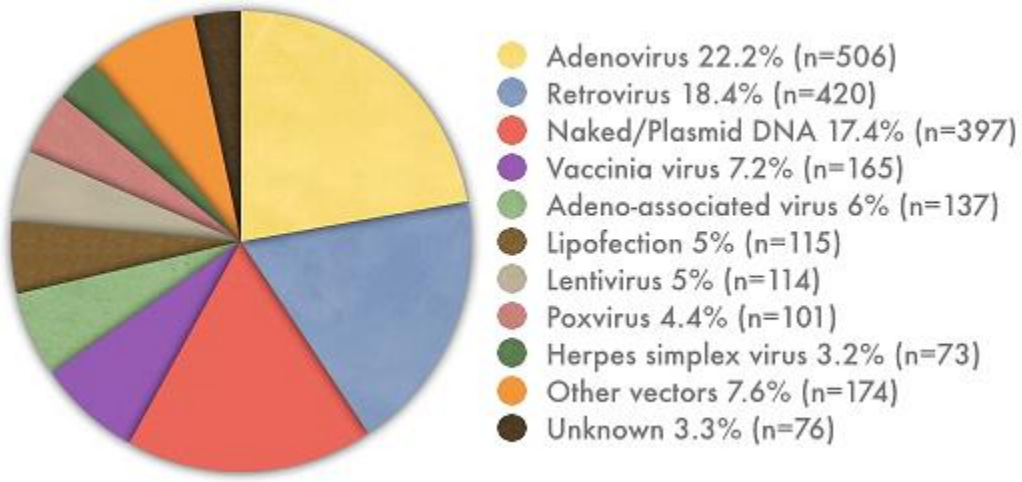

Figure 2: Methods used in gene therapy clinical trials worldwide [12].

Viruses are infectious agents that replicate by infiltrating host cells and using their cellular organization for the expression of viral genes. In gene therapy, viral genes that lead to viral replication and host cell toxicity are replaced with therapeutic genes. The outcome is a virus that uses its highly evolved infection pathway to deliver therapeutic genes to cells. However, the use of viral-based delivery vehicles or viral vectors has major disadvantages [13]. One issue is the inflammatory response that can ensue if a host immune system is triggered by the presence of foreign viral vectors. Another problem is the difficulty in targeting specific cell types, which can lead to unwanted gene delivery. In addition, viral vectors derived from retroviruses can lead to insertional mutagenesis, the integration of DNA that disrupts a sequence. This could induce cancer if the sequence is linked to malignancy. Furthermore, the production of viral vectors is typically complex and expensive.

Liposomes are the most prevalent non-viral nucleic acid delivery vehicles. They are vesicles formed by the self-assembly of lipid molecules, which are composed of hydrophilic heads and hydrophobic tails. When dissolved, lipid molecules arrange themselves into lipid

bilayer membranes to maximize energetically favorable interactions. Eventually, these bilayer membranes curve to close the edges and form liposomes (Figure 3) [14]. In gene therapy, artificial liposomes are prepared using cationic lipids that can interact with the negatively charged backbone of nucleic acids to form complexes with genetic molecules. These complexes can be used to efficiently deliver the genetic molecules because liposomes easily fuse with the lipid bilayer membrane of cells [15]. Like viruses, the use of liposomes for delivery has its limitations [11]. For example, some liposomes have been shown to be toxic. They are also limited by a low efficiency of loading.

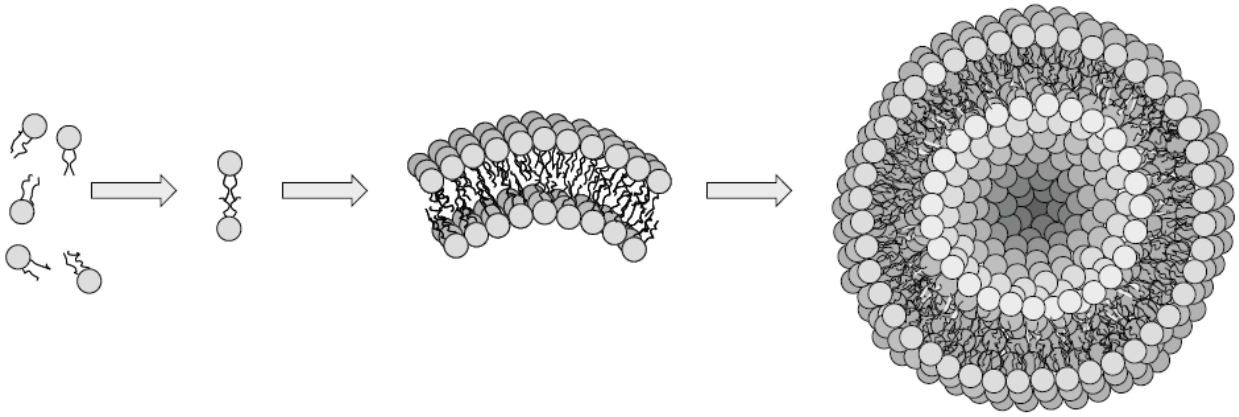

Figure 3: Lipid molecules in solution form bilayer membranes then liposomes [14].

The delivery of therapeutic RNA via exosomes represents a feasible alternative to conventional delivery methods since it does not share the shortcomings that limit their clinical application in gene therapy. This approach seeks to exploit the intrinsic function of exosomes in intercellular communication and harness their natural ability to transport RNA. A crucial feature of exosomes that makes them suitable for RNA delivery applications is their size (40-100 nm), which is on the order of nanoparticles [16]. Particles circulating within the blood stream are

cleared by phagocytotic cells of the reticuloendothelial system if they are too large (250-1000 nm). On the other hand, particles that are too small (1-30nm) will leak into surrounding tissue through extravasation. Exosomes are thus just the right size to avoid these problems and maximize circulation. In addition, exosomes are naturally derived and present in many biological fluids, suggesting they are safe *in vivo*.

A study in 2011 was the first proof-of-concept for exosomes in gene therapy [17]. Dendritic cells were collected from mice and engineered to express a brain targeting peptide connected to a protein found in exosome membranes. Consequently, exosomes derived from these dendritic cells carried the targeting peptide in their membranes. These exosomes were loaded with siRNA using electroporation and administered intravenously to mice. The researchers demonstrated that the targeting peptide successfully directed exosomes to the brain. Furthermore, exosomes loaded with therapeutic siRNA were shown to reduce expression of a protein that is involved in Alzheimer's disease, a neurodegenerative condition. This study is important because it offers insight into the feasibility of using exosomes in gene therapy. Not only were the exosomes capable of protecting the siRNA cargo, but they specifically delivered it to the organ of interest. The exosomes also proved to be immunologically inert because they did not induce an immune response.

The objective of the research presented in this thesis was to investigate the transfection ability and cytotoxicity of exosomes. Transfection is the introduction of genetic molecules into cells. It can be either stable or transient. In stable transfection, the genetic molecules are assimilated into the genome of cells. On the other hand, in transient transfection, the genetic molecules are only briefly present in cells. Since exosomes are limited to transporting RNA, they can only be used for transient transfection. In this research, exosomes were used to transfect cells

with mRNA for the purpose of cell reprogramming, a gene therapy approach in which transient transfection is sufficient [18].

Cellular differentiation is affected by chemical and physical conditions [19]. Lineage-specific transcription factors determine cell types by influencing gene expression. Differentiation was considered to be an irreversible process. However, a major breakthrough in 2006 established that fully differentiated cells could be reprogrammed using transcription factors [20]. Specifically, these researchers demonstrated that the introduction of four transcription factors into mouse fibroblasts successfully induced them into pluripotent stem cells. Another study in 2010 showed that transcription factors could be used to reprogram differentiated cells directly into a different cell type, bypassing the pluripotent cell intermediate [21]. In this case, mouse fibroblasts were directly reprogrammed into neurons by expression of a combination of three transcription factors, *Ascl1*, *Brn2* and *Myt1l* (ABM).

The research presented in this thesis uses exosomes to transfect mouse fibroblasts with ABM mRNA. ABM was chosen because it is a well-established model for the reprogramming of fibroblasts into neurons. This research also evaluates the cytotoxicity of exosomes compared to that of a commercial *in vitro* liposome formulation.

Methods

Plasmid Preparation

Ascl1-GFP, Brn2-RFP and *Myt1l-CFP* pCAGGS plasmids were obtained from Addgene. E. coli were grown at 37 ˚C for 16-18 hours in autoclaved Luria-Bertani (LB) medium and ampicillin (.1%). The cells were harvested by centrifugation (4,000 × g, 30 min, 4 ˚C). A QIAfilter Plasmid Maxi Kit (Qiagen) was used to extract the plasmid DNA. First, alkaline lysis buffers were added and the lysates filtered out. The remaining solution was passed through another filter where the DNA was bound, washed and eluted. Isopropanol was added to precipitate the DNA. Then, Tris-EDTA (1X TE) buffer was used to resuspend the DNA pellet. Finally, a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) was used to quantify DNA concentration. The plasmids were stored at -20 ˚C.

Bulk Electroporation

Bulk electroporation (BEP) is a physical method of transfection. It works by applying an electric field to a solution of cells and genetic molecules. The electric field increases the permeability of the cells' membranes, allowing the genetic molecules to diffuse intracellularly [22].

BEP was used to transfect mouse embryonic fibroblasts (MEFs) with the ABM plasmids. This was accomplished using a Neon Transfection System (Invitrogen). A suspension of MEFs (1×10^6 cells in 100 µL) was mixed with a combination of plasmids (0.05 µg/µL) at a molar ratio of 2:1:1 (*Ascl1*:*Brn2*:*Myt1l*). The resulting mixture was loaded into the Neon pipette tip. Then, the pipette was plugged into the Neon transfection device in a tube containing electrolytic buffer. Electroporation was conducted using the manufacturer's recommended setting for MEFs (one 30 ms pulse of 1350 V). Finally, the cells were centrifuged and incubated in exosome-

depleted media. Electroporation settings other than the manufacturer's recommendation were attempted but resulted in a significant decrease in cell viability.

Exosome Isolation

Exosomes were isolated from the supernatant of electroporated MEFs 48 hours after they were transfected with ABM. This was accomplished using ExoQuick-TC (System Biosciences), a polymer-based exosome precipitation kit. The supernatant was first centrifuged (3,000 × g, 15 min, 4 ˚C) to remove cells and cell debris. ExoQuick-TC exosome precipitation solution was added at a volume ratio of 1:3 (precipitation solution: supernatant). The resulting solution was refrigerated overnight at 4 ˚C. Then, exosomes were isolated from the solution by centrifugation (1,500 × g, 30 min, 4 ˚C).

RNA Isolation

A *mir*Vana miRNA Isolation Kit (Life Technologies) was used to isolate RNA from exosomes. The exosomes were first lysed using the Lysis/Binding Solution (500 µL). Then miRNA Homogenate Additive (50 µL) was added. RNA extraction was achieved using Acid-Phenol: Chloroform (500 µL) and centrifugation (10,000 × g, 5 min) to separate the aqueous and organic layers. The extracted aqueous layer was mixed with ethanol (100%, 625 µL). A filter was used to bind and wash the RNA. Finally, the RNA was eluted using DEPC-treated water and stored at -80 ˚C.

To isolate RNA from MEFs, TRIzol Reagent (Life Technologies) (300 µL) was used to lyse the cells. RNA was then extracted using chloroform (60 µL) and centrifugation (12,000 × g, 15 min, 4 ˚C) to separate aqueous and organic layers. Isopropanol was used to precipitate the RNA in the aqueous layer, and the solution was centrifuged (12,000 × g, 10 min, 4 ˚C). The

pellet was washed with ethanol. After the RNA pellet was left to air dry, it was resuspended in DEPC-treated water and stored at -80 ˚C.

NanoDrop 2000 was used to determine RNA concentration.

Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) was used to produce cDNA from the RNA isolated previously. Each sample was prepared using a High Capacity cDNA kit (Invitrogen) by mixing reverse transcriptase buffer (10X, 2 µL), dNTPs (100 mM, 0.8 µL), Random Primers (2 µL), reverse transcriptase (1 µL), RNase inhibitor (1 µL) and the RNA in nuclease-free water (100 ng, 13 µL). A Veriti Thermal Cycler (Applied Biosystems) was loaded with the samples and run according to the manufacturer's protocol.

cDNA generated from RT-PCR was then used in real-time PCR (qPCR) to quantify the ABM mRNA originally present. Each sample was prepared by mixing iQ SYBR Green Supermix (Bio-Rad) (5 µL), forward primer (0.2 µL), reverse primer (0.2 µL), nuclease-free water (3.6 µL) and the cDNA (1 µL). The samples were then loaded into a StepOnePlus Real-Time PCR System (Applied Biosystems), which was subsequently run according to the manufacturer's protocol.

Liposome Preparation

Liposomes were prepared using the commercial Lipofectamine 2000 Reagent (Life Technologies), a cationic liposome formulation used for *in vitro* applications. The liposomes were produced according to the manufacturer's protocol by mixing the Lipofectamine reagent with a combination of plasmids (0.05 µg/µL) at a molar ratio of 2:1:1 (*Ascl1*:*Brn2*:*Myt1l*). For the cytotoxicity experiment, this mixture was incubated with MEFs (3×10^5 cells per well) for 48 hours.

Results

Fluorescence microscopy was used to image MEFs 48 hours after transfection with ABM plasmids using BEP (Figure 4). The green fluorescence resulted from expression of the green fluorescent protein (GFP) tagged to *Ascl1* plasmids. This indicates that the BEP transfection of MEFs with *Ascl1* plasmids was successful.

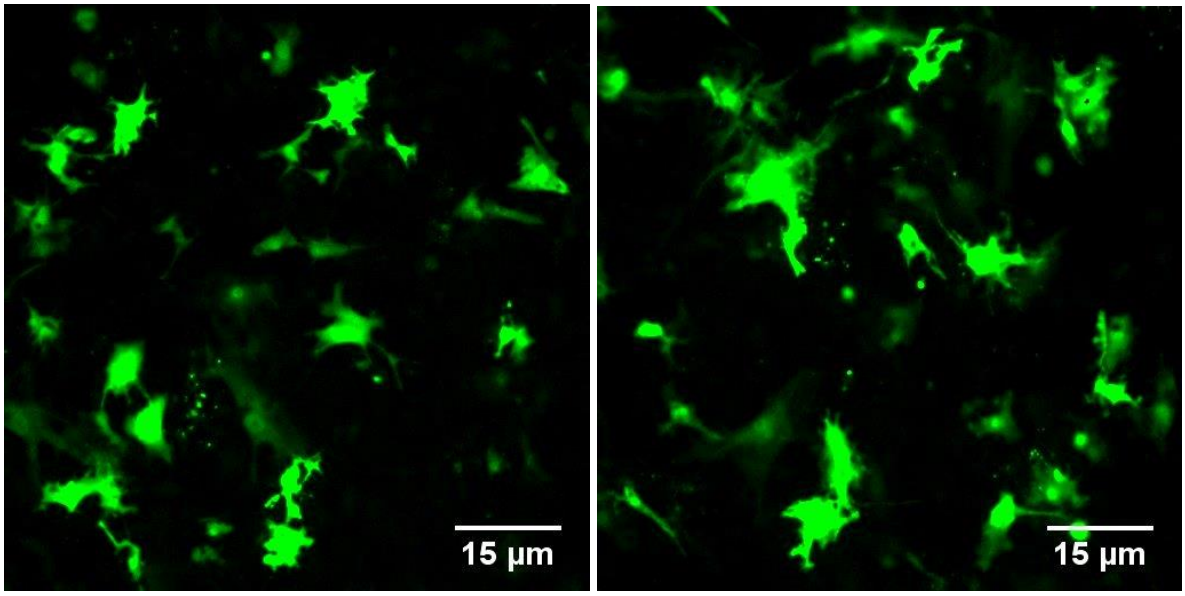

Figure 4: Green fluorescence due to expression of GFP tag on *Ascl1* plasmids.

RNA was isolated from the MEFs 48 hours after transfection with ABM plasmids. The amount of ABM mRNA was quantified using qPCR after RT-PCR (Figure 5). There was an increase in *Ascl1* mRNA compared to untreated control MEFs. However, *Brn2* and *Myt1l* mRNA content was found to be lower than control.

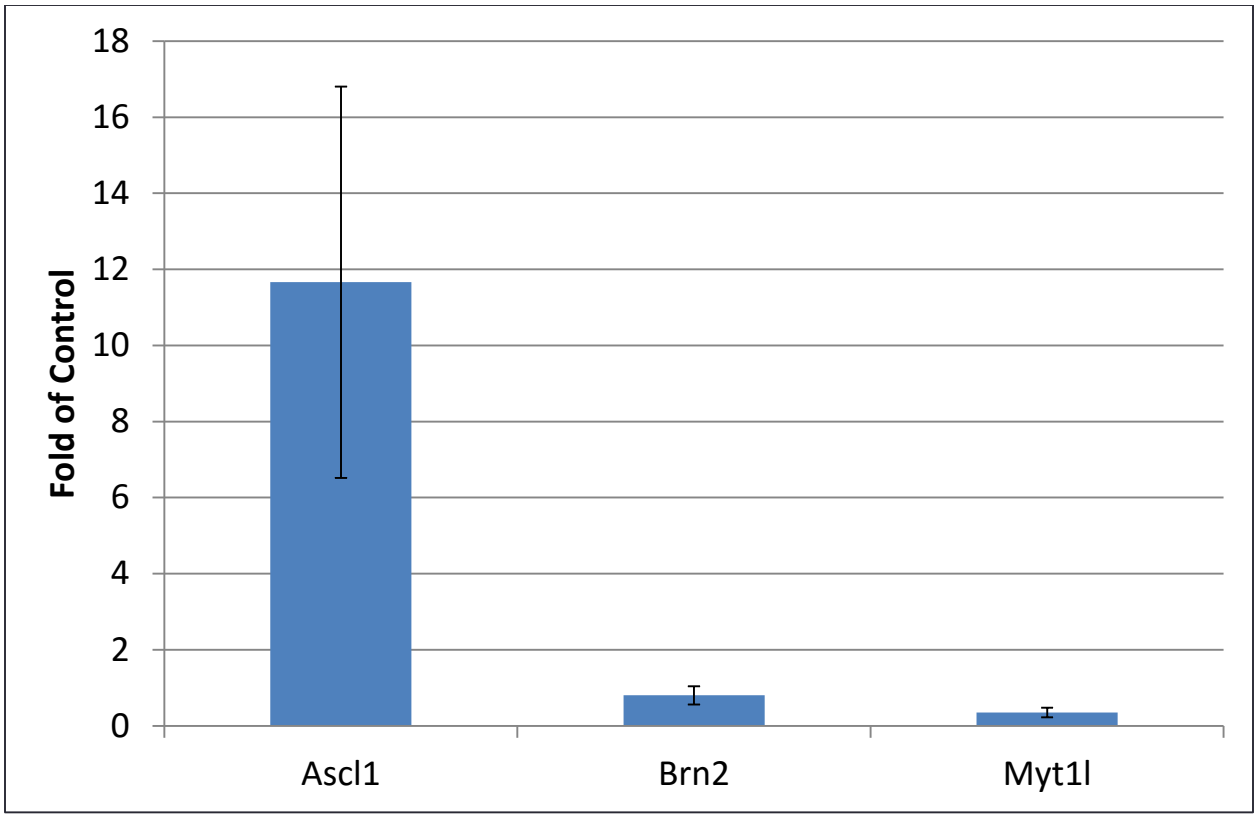

Figure 5: qPCR of ABM mRNA from MEFs 48 hours after transfection with ABM plasmids using BEP.

Exosomes were derived from the MEFs 48 hours after they were transfected. RNA was isolated from these exosomes and qPCR was used to quantify their ABM mRNA content (Figure 6). The *Ascl1*, *Brn2* and *Myt1l* mRNA content of the exosomes showed an increase from that of control exosomes derived from untreated MEFs.

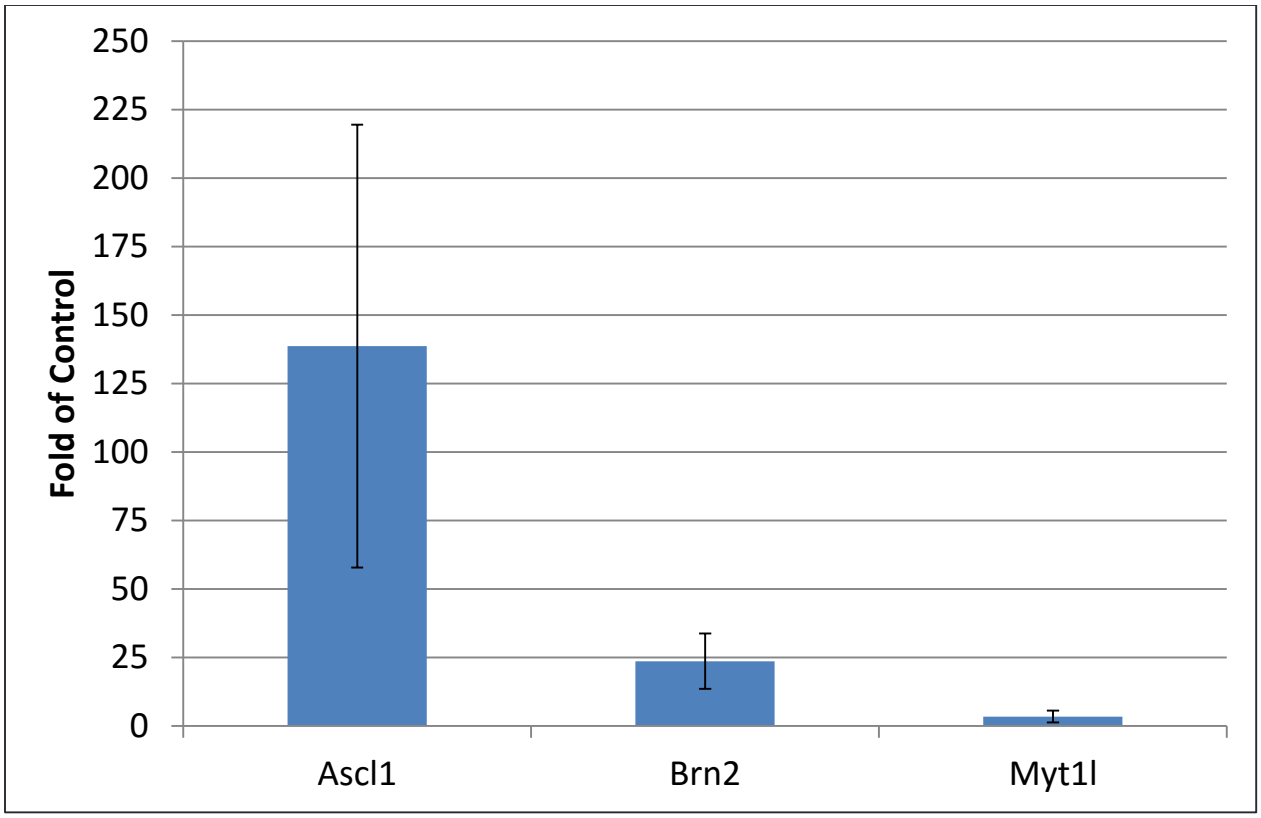

Figure 6: qPCR of ABM mRNA from exosomes.

These exosomes were incubated with a fresh set of MEFs for 48 hours. To determine whether the exosomes transfected the cells, RNA was isolated from the MEFs and qPCR was used to quantify the cells' ABM mRNA content (Figure 7). It was found that exosomes transfected the MEFs because the cells showed an increase in ABM mRNA compared to untreated cells.

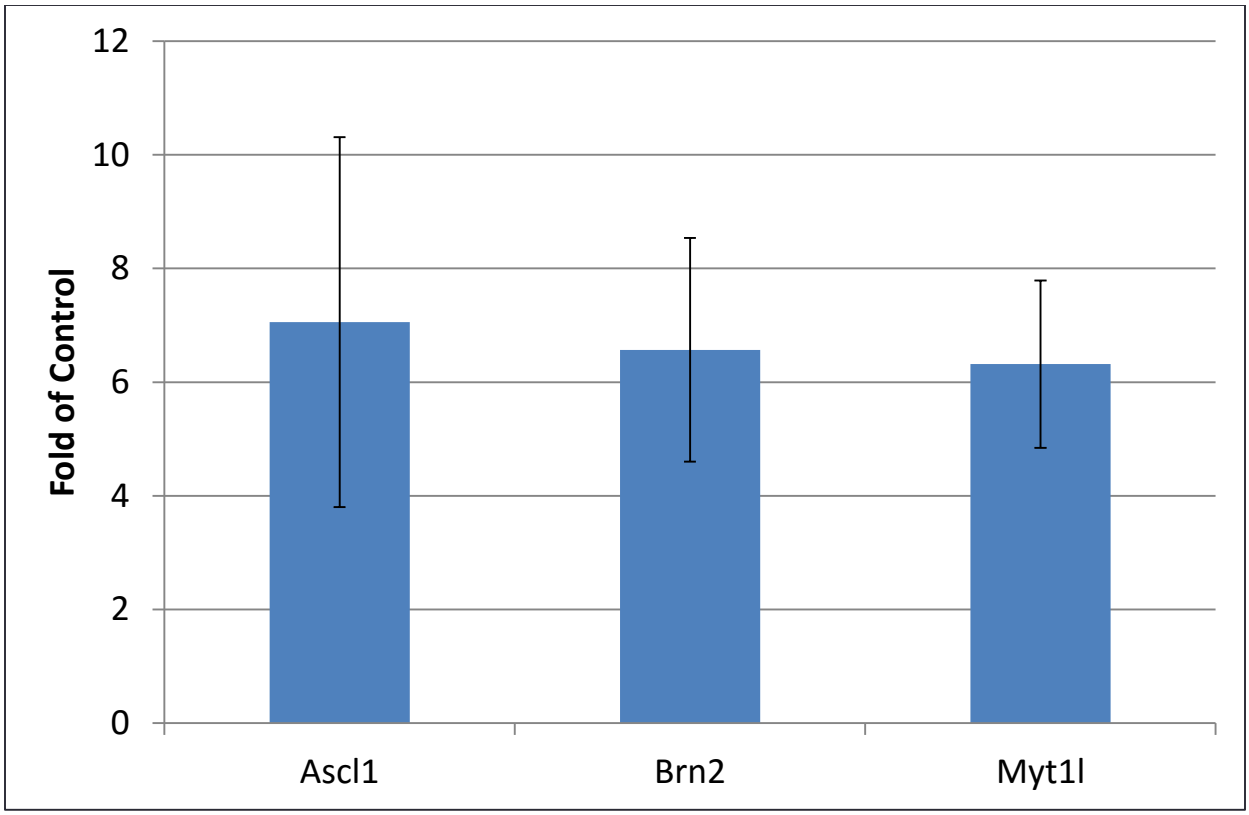

Figure 7: qPCR of ABM mRNA from MEFs 48 hours after incubation with exosomes derived from transfected MEFs.

To investigate exosome cytotoxicity, MEFs were incubated with exosomes derived from transfected MEFs and ABM plasmid-loaded liposomes prepared using Lipofectamine reagent. After 48 hours, MEFs incubated with exosomes demonstrated a 94% viability of control, while those incubated with liposomes had a 28% viability (Figures 8 & 9).

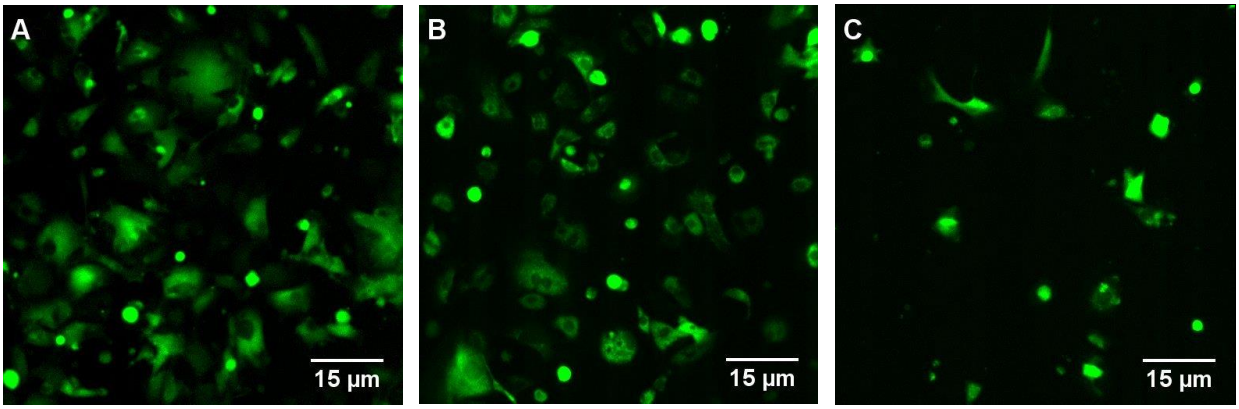

Figure 8: (A) Untreated MEFs. MEFs incubated with (B) exosomes and (C) liposomes for 48 hours.

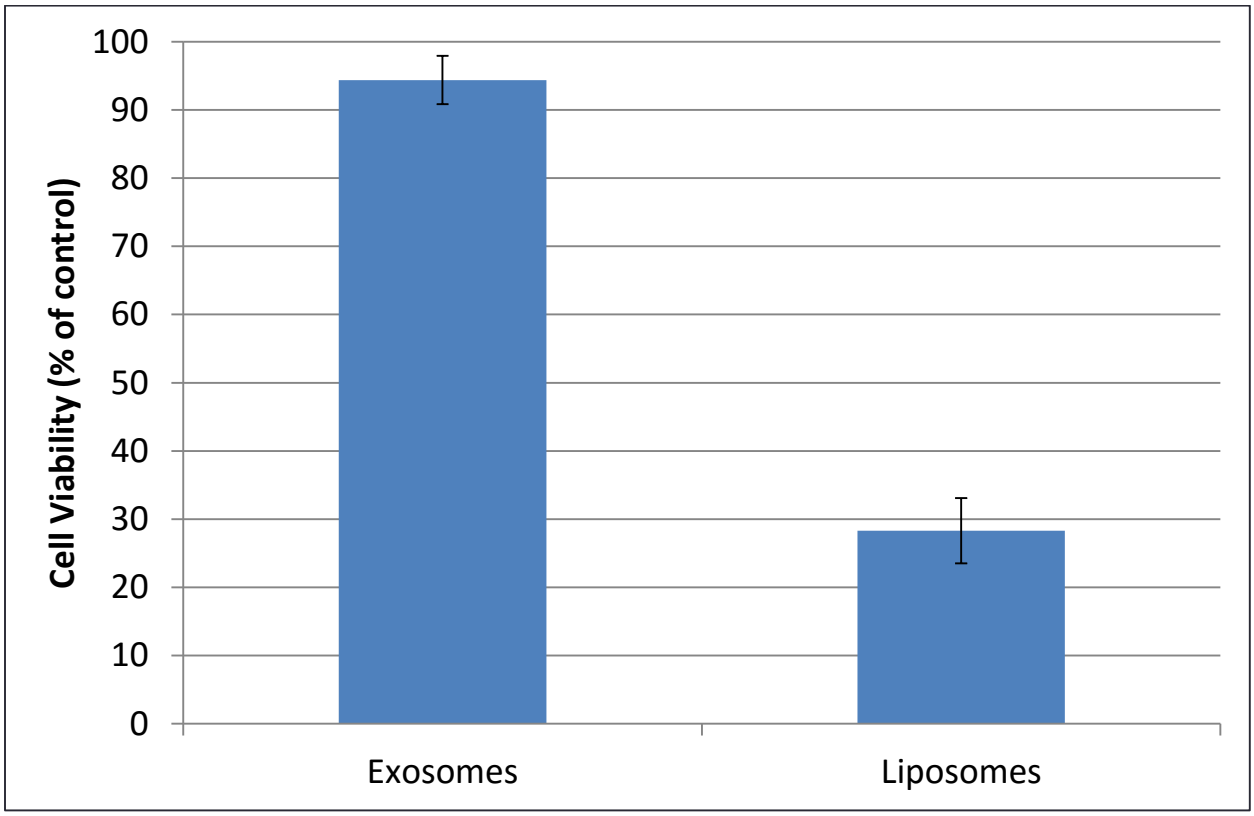

Figure 9: MEF viability after incubation with exosomes and liposomes for 48 hours.

Discussion

The low quantities of *Brn2* and *Myt1l* mRNA in electroporated MEFs (Figure 5) and their exosomes (Figure 6) could be attributed to an unsuccessful transfection of the MEFs with *Brn2* and *Myt1l* plasmids. Since it is diffusion dependent, BEP is known to cause stochastic transfections [23, 24, 25, 26]. Additionally, the *Brn2* (1.3k bp) and *Myt1l* (3.5k bp) gene inserts are much larger than the *Ascl1* (0.7 k bp) gene insert, and as such, their plasmids would have diffused more slowly into cells during BEP. Future work will investigate whether electroporated MEFs were actually transfected with the *Brn2* and *Myt1l* plasmids. Another possible explanation for the low quantities of *Brn2* and *Myt1l* mRNA in electroporated MEFs and their exosomes could be gene silencing of *Brn2* and *Myt1l*. This would also account for the observed decrease in *Brn2* and *Myt1l* mRNA in cells after electroporation (Figure 5). Future work will therefore examine whether gene silencing was affecting the *Brn2* and *Ascl1* mRNA content.

Exosomes derived from electroporated MEFs successfully transfected cells *in vitro* with ABM mRNA (Figure 7). However, the cells' mRNA increased to a similar extent for all three transcription factors, despite the fact that the exosomes used were loaded with considerably lower proportions of *Brn2* and *Myt1l* mRNA than *Ascl1* mRNA (Figure 6). Future work will focus on determining a cause for these strange results. In addition, future work will investigate whether MEFs transfected with exosomes express the ABM transcription factors and undergo reprogramming into neurons.

The cytotoxicity experiment showed that cell viability is minimally impacted upon incubation with exosomes. It also demonstrated that exosomes are less cytotoxic than a more conventional method for the delivery of nucleic acids. However, it must be noted that the liposomes used were prepared using a commercial Lipofectamine reagent intended for *in vitro*

transfection. As such, this formulation was designed to maximize transfection efficiency rather than limit cytotoxicity. Future work will compare the cytotoxicity of exosomes with liposome formulations meant for *in vivo* applications which are less cytotoxic than Lipofectamine.

Conclusion

Exosomes are natural intercellular transporters of RNA that are involved in various functions *in vivo*. Due to their intrinsic properties, exosomes could represent a more effective method for RNA transfection in gene therapy than conventional nucleic acid delivery vehicles. The research presented in this thesis used exosomes for the *in vitro* transfection of fibroblasts with mRNA for ABM, a combination of transcription factors that reprograms fibroblasts into neurons. Furthermore, the cytotoxicity of exosomes was investigated and found to be less than that of a common *in vitro* transfection method.

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